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Analysis of genetic diversity and relationships in East African banana germplasm

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Abstract The genetic diversity and phylogenetic relationships of 29 East African highland banana (*Musa* spp.) cultivars and two outgroup taxa, *M. acuminata* Calcutta 4 and Agbagba were surveyed by RAPD analysis. A genetic similarity matrix was established based on the presence or absence of polymorphic amplified fragments. Phylogenetic relationships were determined by UPGMA cluster analysis. RAPDs showed that the highland bananas are closely related with a narrow genetic base. Nevertheless, there were sufficient RAPD polymorphisms that were collectively useful in distinguishing the cultivars. The dendrogram was divisible into a major cluster composed of all the AAA highland banana cultivars and Agbagba (AAB) and a minor cluster consisting of Kisubi (AB), Kamaramasenge (AB) and Calcutta 4 (AA). Several subgroups are recognized within the major cluster. RAPD data did not separate beer and cooking banana cultivars. Our study showed that RAPD markers can readily dissect genetic differences between the closely related highland bananas and provide a basis for the selection of parents for improvement of this germplasm.

Keywords *Musa* · Bananas · Cluster analysis · Genetic diversity · RAPD markers

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Introduction

Banana is a highly valued food crop in the highlands of East Africa, especially in Burundi, Kenya, Rwanda, western Tanzania, Uganda, and eastern Congo (Ddungu 1987; Sebasigari 1987). The region records one of the highest consumption figures for bananas in the world at over 300 kg per capita per year. Banana is the most extensively grown crop in Uganda, occupying about 40% of the arable land (Horry et al. 1998a). These bananas grow in high rainfall regions at altitudes between 1200 and 1900 m above sea level and are known as East African highland bananas (EAHB). Two main types of bananas, based on the use of the fruits, are recognized in this region (Sebasigari 1987): bananas that are used for boiling or steaming (cooking banana) and those used for the preparation of beverages (beer bananas). Although the highland bananas have an AAA genome composition (Shepherd 1957), they are distinctly different from the dessert or sweet bananas that have similar genomes (AAA) and from the plantains (AAB) and cooking bananas (ABB) that are cultivated mainly in the humid lowlands of West and Central Africa. It is estimated that there are between 45 and 70 highland banana cultivars in East Africa (Baker and Simmonds 1952; Shepherd 1957) since a number of local names may exist for the same cultivar. Many cultivars cannot be easily distinguished on the basis of their morphology, especially if they are closely related.

Information on the genetic diversity and phylogenetic relationships within the East African banana germplasm is scarce. These relationships are essential to develop an efficient breeding program by providing basic information for breeders, such as the selection of suitable material for new cultivar development. Advances in *Musa* breeding have established that crossing of divergent genotypes and subsequent selection of improved hybrids are important steps for the production of new banana cultivars (Ortiz and Vuylsteke 1996).

Genetic diversity in plants can be estimated in several ways. The RAPD (random amplified polymorphic DNA, Williams et al. 1990) technique has been used extensive-

ly for this and other research purposes since data can be generated faster and with less labor than other methods such as restriction fragment length polymorphisms (RFLPs), single sequence repeat or microsatellites (SSRs) and amplified fragment length polymorphisms (AFLPs). RAPDs have been used to assess genetic variation and phylogenetic relationships in many plants including *Citrus* (Federici et al. 1998), *Phaseolus vulgaris* (Briand et al. 1998), *Prunus* (Shimada et al. 1999) and *Vitis* (Vidal et al. 1999).

The study reported here aimed at using RAPD markers to: (1) assess the level of genetic diversity in EAHB germplasm; (2) construct a dendrogram to show relationships amongst the genotypes; (3) compare this scheme with shared morphological features of the plants; and (4) determine the genome composition of the plants.

Materials and methods

Plant material

The plant material (Table 1) consisted of 29 EAHB cultivars and two outgroup taxa, *Musa acuminata* subsp. *burmanicoides*, Calcutta 4 (AA), hereinafter referred to as C4, and the plantain Agbagba (AAB). The sample included both beer and cooking bananas. These plants were randomly selected from the germplasm collection of the International Institute of Tropical Agriculture, Onne Research Station, southeastern Nigeria.

Genomic DNA isolation and RAPD analysis

Total genomic DNA was extracted from fresh leaf material using a modified CTAB procedure as reported by Crouch et al. (1998). The DNA samples were diluted for PCR (polymerase chain reaction) to a final concentration of 0.2 µg/µl. Eighty 10-mer oligonucleotides (kits A, B, C, D) from Operon Technologies (USA) were used as single primers in PCR reactions. Amplification reaction volumes were 25 µl, each containing 0.2 µg DNA, 2.0 m*M* $MgCl_2$, 0.2 m*M* each dNTPs, 1.25 U *Taq* polymerase (Advanced Biotechnologies, Surrey, UK) and 1.2 µ*M* primer in a reaction buffer containing 75 m*M* TRIS-HCl, pH 9.0 and 20 m*M* $(NH_4)_2SO_4$. Amplifications were performed in a Perkin Elmer Cetus 9600 Thermal Cycler with the following temperature cycles: an initial 3-min denaturation at 94°C, followed by 35 cycles of 50 s at 94°C, 50 s at 40°C and 1.5 min at 72°C. The final extension step was done at 72°C for 7 min. Amplification products were resolved by electrophoresis on 1.5% agarose gels in 1× TBE buffer. DNA gels were stained in ethidium bromide and then photographed under UV light using Polaroid 667 film. Molecular size markers included DNA fragments of the 100-bp and 1-kb ladder (Life Technologies, USA).

Hybridization analysis

DNA gels with amplification products were transferred to nylon membranes as described by Reed and Mann (1985). Fragments used as probes were excised from duplicate gels and the DNA extracted with the Agarose Gel DNA Extraction Kit (Boehringer Mannheim, Mannheim, Germany). Labeling of fragments, hybridization and detection were done with the ECL direct nucleic acid labeling and detection system (Amersham Int, Buckinghamshire, England).

Table 1 Cultivars, banana type, ploidy and genome composition of plants used in genetic diversity studies of East African banana germplasm

Serial number	Accession name	Type	Ploidy	Genome composition
1	Kibungo 1	Cooking	3x	AAA
2	Igisahira Gisanzwe	Cooking	3x	AAA
3	Nsira	Cooking	3x	AAA
4	Kitawira	Cooking	3x	AAA
5	Makara	Beer	3x	AAA
6	Isha	Beer	3x	AAA
7	Kisubi	Dessert	2x	AB
8	Ikigeregere	Beer	3x	AAA
9	Nshika	Beer	3x	AAA
10	Ikimaga	Cooking	3x	AAA
11	Igitsiri	Beer	3x	AAA
12	Mbawzirume	Cooking	3x	AAA
13	Intokatoke	Beer	3x	AAA
14	Ingagara	Cooking	3x	AAA
15	Igihuni	Cooking	3x	AAA
16	Inkira	Beer	3x	AAA
17	Inyoya	Cooking	3x	AAA
18	Kagera	Cooking	3x	AAA
19	Kamaramasenge	Dessert	2x	AB
20	Indemera y' Imbihire	Beer	3x	AAA
21	Imbogo	Cooking	3x	AAA
22	Nakitengwe	Cooking	3x	AAA
23	Bakurura	Cooking	3x	AAA
24	Ingoromoka	Cooking	3x	AAA
25	Mbirabire	Cooking	3x	AAA
26	Ingumba y' Inyamunyo	Cooking	3x	AAA
27	Intama	Cooking	3x	AAA
28	Ingarama	Cooking	3x	AAA
29	Intariho	Cooking	3x	AAA
30	Calcutta 4	Wild	2x	AA
31	Agbagba	Plantain	3x	AAB

Flow cytometry and genome composition

The ploidy level of each plant was determined by flow cytometry and their genome composition determined by using RAPD markers that are specific to the A and B genomes as described by Pillay et al. (2000).

Data analysis

Only clear and unambiguous polymorphic bands were scored for the analysis. RAPD fragments for each primer were scored as present (1) or absent (0). Bands with the same migration distance were considered homologous (see hybridization analysis). A pairwise similarity matrix was computed and analyzed with NTSYS (Rohlf 1994) version 1.80 using the simple matching coefficient (Sokal and Michener 1958). The similarity matrix was used to construct a dendrogram by the unweighted pair-group method with arithmetical averages (UPGMA).

Results

RAPD data

Our RAPD technique has been optimized for banana and produced amplified fragments varying from 150 to 3000 bp in size. Figure 1A illustrates the typical level of polymorphisms observed in a sample of the banana cultivars, which was generally low among the EAHB cultivars. To determine whether fragments with similar migration patterns were homologous, we transferred randomly selected gels to membranes and subsequently selected specific

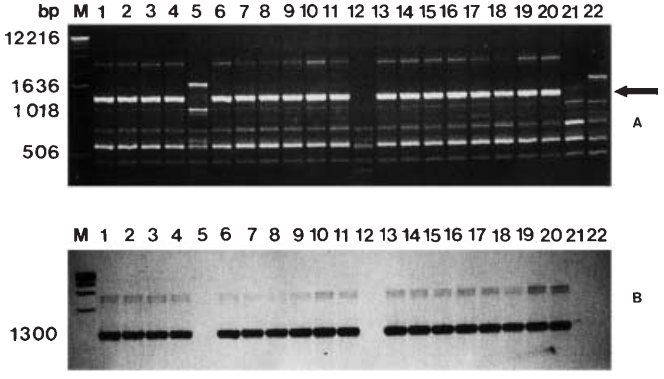

Fig 1 A RAPD profiles of DNA from 22 cultivars of East African bananas using primer D10. **B** Southern hybridization patterns of the RAPD profiles in **A** when the 1300-bp fragment was used as a probe. *M* represents molecular-weight markers

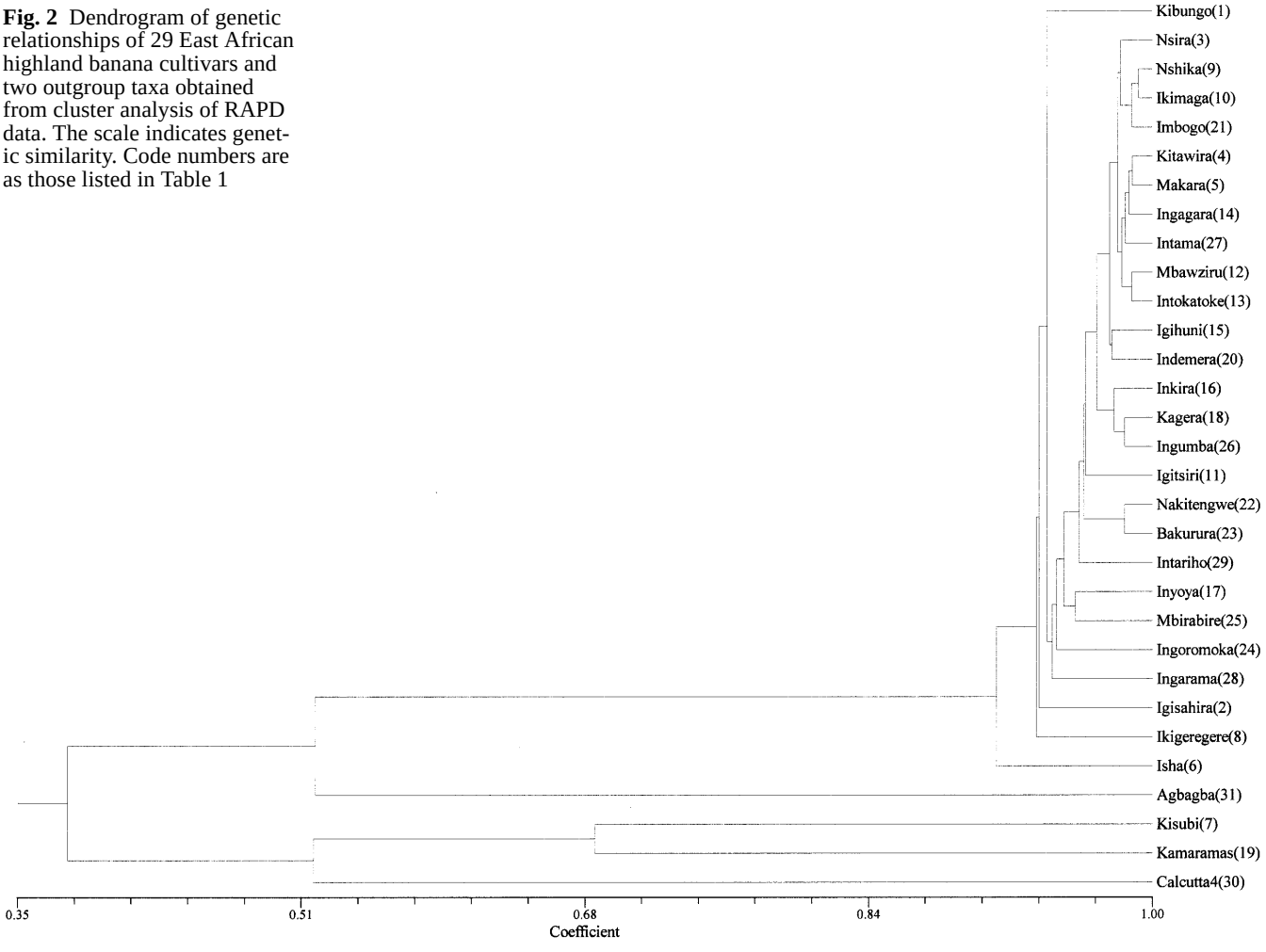

Fig. 2 Dendrogram of genetic relationships of 29 East African highland banana cultivars and two outgroup taxa obtained from cluster analysis of RAPD data. The scale indicates genetic similarity. Code numbers are as those listed in Table 1

fragments as hybridization probes. Figure 1B illustrates the Southern hybridization results when the presence or absence of the 1300-bp fragment was considered for analysis.

The genetic similarity coefficients for the 31 clones ranged from 0.30 to 0.98. The UPGMA analysis made it possible to discriminate all of the genotypes of this study. The dendrogram (Fig. 2) showed a clear distinction into a major and minor cluster. The major cluster was composed of all the AAA genotypes and the plantain Agbagba that clustered with about 52% similarity. Within the major cluster, two beer cultivars, Isha and Ikigeregere, and two cooking cultivars, Igisahira and Kibungo 1, were quite distinct and appeared as separate entities. The remaining cultivars formed a single group with about 95.5% similarity. Several subgroups are recognizable within this group (Fig. 2). The largest subgroup with 97.8% similarity was composed of Nsira, Nshika, Ikimaga, Imbogo, Kitawira, Makara, Ingagara, Intama, Mbawsirume and Intokatoke. Within this subgroup, the closest relationship at approximately 98.6% similarity occurred between the beer bananas Nshika and Ikimaga. The beer banana Makara paired with Kitawira, while Intokatoke paired with Mbwasirume. Igihuni and Indemera y' Imbihire clustered with the largest subgroup with about 97.4% similarity. Other subgroups included (1) Inkira, Kagera and Ingumba y' Inyamunyo, (2) Nakitengwa and Bakurura and (3) Inyoyo and Mbirabire. Cultivars Igitsiri, Intariho, Ingoromoka and Ingarama were quite distinctive and appeared as separate entities. The minor cluster consisted of Kisubi, Kamaramasenge and C4. Kisubi and Kamaramasenge clustered at 68% similarity, while C4 clustered with the former cultivars at 52% similarity.

Ploidy analysis and genome composition

With the exception of the diploids, Kisubi and Kamaramasenge, with AB genomes, the other highland banana cultivars were triploids with AAA genomes (Table 1).

Discussion

Genetic diversity

This study was conducted to determine the extent of genetic diversity in a sample of the highland banana germplasm of East Africa, based on the RAPD marker system that makes use of arbitrary primers to amplify random DNA sequences in the genome (Williams et al. 1990). Until recently, morphology was used extensively to determine relationships among plants. This was also the case in *Musa* in which morphological and agronomic characters were used to assess genetic relationships (Ortiz 1997; Ortiz et al. 1998; Karamura 1998). Morphological markers are limited in number and they do not often reflect genetic relationships because of interaction with the environment, epistasis and the largely unknown genetic control of the traits (Smith and Smith 1989). In contrast, DNA markers are found in abundance and are not influenced by the environment or developmental stage of a plant, making them ideal for genetic relationship studies. This is the first study that has made use of molecular markers to examine genetic diversity in East African bananas.

With the exception of Kisubi, Kamaramasenge and the outgroups C4 and Agbagba, RAPD analysis suggests that the East African bananas used in this study are closely related with a narrow genetic base. Nevertheless, the RAPD polymorphisms are collectively useful in distinguishing the cultivars. Champion (1970) suggested that these cultivars evolved from a very ancient single introduction. Hence, the variation in this germplasm is the result of somatic mutations and subsequent preferential cultivation of the mutants (Simmonds 1966). The contrasting physical features and climates of East Africa and the social backgrounds of the region may have also played a role in the diversification of the different clones (Karamura 1998). East Africa is regarded as the secondary center of diversity for the *Musa* AAA group (Swennen et al. 1995), as a result of the accumulation of variation in this germplasm. Genetic variability and cultivar development in other clonally propagated plant species such as citrus have also arisen through frequent somatic mutations (Fang and Roose 1997; Asins et al. 1999).

There are indications that retrotransposons are responsible for spontaneous mutations in plants (Hirochika 1995). Asins et al. (1999) have suggested that retrotransposon activity might be, at least in part, involved in genetic variability in sweet orange cultivars. Retrotransposons have been identified in banana (Balant-Kurti et al. 1999; Khayat et al. 1999). Although the evolutionary role of these transposons has not been fully investigated in *Musa*, their involvement in inducing genetic variability as demonstrated in citrus cannot be discounted.

Low levels of genetic diversity in a group of closely related organisms are generally ascribed to factors such as domestication, a genetic bottleneck (temporary reduction in population size) or founder effects (establishment of a population by a single or few individuals). There appears to be no documented evidence to support the latter two effects, except Champion's (1970) hypothesis on a single ancestor. Consequently, we suggest that domestication could also be a major factor responsible for the reduced variability in the highland bananas. Bananas were introduced into East Africa between 0–600 AD (Simmonds 1966) and, therefore, have a long and intense history of cultivation (De Langhe 1961). Limited genetic diversity has also been recorded for many other domesticated species including beets (Jung et al. 1993), barley (Saghai-Maroof et al. 1994) and lentils (Ferguson et al. 1998).

The low level of DNA diversity contrasts with the high level of morphological variability present in these plants. Probable reasons for this discordance are the following. The RAPD primers did not anneal to areas of the genome responsible for the morphological variation, re-

sulting in non-random sampling of the genome and an insufficient number of polymorphisms. The morphological variation is the result of genotype × environmental interaction. There are many reports of environmental effects on banana. For example, Daniells (1990) reported marked environmental effects on plant phenotypes within the Cavendish sub-group.

The a priori inclusion of the wild diploid banana, C4 (AA), and the plantain, Agbagba (AAB), as outgroup taxa provides useful insights for breeding. C4 is a source of useful gene(s) for resistance to black Sigatoka, a devastating fungal disease caused by *Mycosphaerella fijiensis*. Consequently, it has been used widely as a male parent in most breeding programs as a source of resistance genes. This study showed that C4 is quite distinct from the East African bananas. Therefore, while it may be a useful source of disease resistance, it is unlikely to have good combining ability with the East African cultivars. Interestingly, this was found to be true when C4 was crossed with East African cultivars (Hartman and Vuylsteke, unpublished data).

The exact origin of the highland bananas has not been unequivocally established. The close genetic similarity and the large number of shared fragments suggest a common origin and subsequent divergence by mutations. Price (1995) suggests that they were introduced directly from South-east Asia where AA and AAA cultivars are common. Although C4 is an AA diploid, our results show a strong distinction between it and the highland bananas, implying that the former may not be involved in the direct ancestry of these plants. Rather, it is more closely associated with the south Indian cultivars Kisubi and Kamaramasenge. The low level of genetic similarity of Agbagba with the highland bananas is probably due to their different genome compositions. It supports the view of Simmonds (1966) that plantains were introduced to Africa independently of the highland bananas.

Comparison with morphology

One of the interesting aspects of this study was the clustering of the two AB diploid dessert cultivars, Kisubi and Kamaramasenge. Field data show that the two cultivars are very similar in morphology and resemble Ney poovan. Kisubi is considered to be a recent introduction directly or indirectly from south India (Stover and Simmonds 1987). Although no information is available on the origin of Kamaramasenge, its close affinity to Kisubi coupled with its literature name of Ney poovan (Sebasigari 1987) suggests a south Indian origin. It is likely that Indian immigrants introduced the south Indian cultivars into East Africa. RAPD data showed that they are quite distinct from the highland bananas (Fig. 2).

Morphological descriptions were available for 20 of the 29 cultivars used in this study. It was possible to compare the clustering based on RAPD profiles with the morphological characteristics of the plants. With a few exceptions, DNA clustering patterns were in general agreement with the shared morphological characteristics of the cultivars. For example, Inyoyo and Mbirabire were scored to be similar in 42 out of 48 morphological descriptors. Similarly, Ingarara and Kitawira in the same clade shared 37 out of 48 characters, while Intama and Kitawira shared 36 characters. In contrast, Makara grouped closer to Kitawira, but these accessions shared only 28 out of 42 characters.

RAPD data corresponded with field data in showing the distinctiveness of some of the varieties. For example, the pseudostem in Intama is characterized by moderate brown blotches, while the pseudostems of most of the other plants in this study have extensive black blotches. Intama is also one of the few plants with erect fingers. Although Igihuni, a cooking cultivar, grouped with Indemera y' Imbihire, a beer cultivar, the former is easily distinguishable in the field by its bright yellow-orange male bud, large persistent male bracts, persistent neutral flowers and styles. Similarly, Igitsiri is recognizable by the extensive and intense black blotching of the pseudostem.

The RAPD profiles were not able to clearly separate the beer and cooking banana cultivars. With the exception of Nshika and Ikimaga that appeared in a sister group relationship, and Isha that appeared alone in a clade, other beer cultivars clustered randomly with cooking cultivars. The two types of bananas share a large number of morphological characteristics (Sebasigari 1987) and cannot be distinguished on the basis of morphology. Only the color and taste of their unripe pulp distinguishes them: cooking bananas have a rather insipid flavor, while beer bananas are bitter (Sebasigari 1987; Karamura 1998). Hybridization, whether natural or artificial has played a role in the evolution of the highland bananas. Continuous gene flow between beer and cooking cultivars that grow sympatrically has probably masked genetic differentiation between the two types of banana. Our inability to differentiate between them may also be due to the limited number of RAPD primers used in this study. Our data do not correspond with those of a phenetic study (Karamura 1998) that showed a clear separation of beer and cooking bananas. The most likely reason for this differential result is that the color and taste of the pulp, key characters that separate beer and cooking types, were included in the 73 characters used by Karamura (1998).

Ploidy and genome composition

Flow cytometry showed that the highland bananas were triploid. Prior to this study, the ploidy level of these plants, and in *Musa* in general, was estimated by various phenotypic traits (Simmonds and Shepherd 1955). Phenotypic traits are influenced by the genotype and cannot be recommended for reliable ploidy estimation in *Musa* (Vandenhout et al. 1995; van Duren et al. 1996). The greater precision of flow cytometry has already proved useful in reassigning ploidy levels in a number of *Musa* cultivars (Horry et al. 1998b). Our RAPD marker system (Pillay et al.2000) also confirmed the genome composition of the highland bananas to be AAA.

The results of this study indicate that RAPD analysis is a reliable and effective means of assaying genetic variation in East African banana. RAPD markers can easily distin-

guish highland banana cultivars, even closely related ones. This study provides a basis for breeders to identify diverse cultivars for breeding. The advantages of the RAPD technique, including its speed, low DNA template requirements and technical simplicity, makes it a convenient tool for detecting genetic variation within this germplasm.

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